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HORMONES – CYTOKINES – SIGNALING

Reactive oxygen species amplify protein kinase C signaling in high glucose-induced fibronectin expression by human peritoneal mesothelial cells

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Reactive oxygen species amplify protein kinase C signaling in high glucose-induced fibronectin expression by human peritoneal mesothelial cells.

Background. We previously demonstrated that high glucose up-regulates fibronectin mRNA and protein expression by human peritoneal mesothelial cells (HPMC) through activation of protein kinase C (PKC). PKC is known to induce cellular reactive oxygen species (ROS) and PKC-dependent activation of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has recently been shown to be responsible, in part, for increased oxidative stress in diabetes. On the other hand, high glucose-induced mitochondrial overproduction of superoxide anion was found to activate PKC. We, therefore, hypothesized that high glucose-induced activation of PKC in HPMC may increase cellular ROS and ROS, in turn, may activate PKC and thus provide signal amplification in high glucose-induced fibronectin up-regulation in HPMC.

Methods. The role of ROS in high glucose- and PKC-induced fibronectin expression was examined by quantification of cellular ROS after stimulation with high glucose and phorbol 12-myristate 13-acetate (PMA), by the effect of hydrogen peroxide (H_2O_2) and PMA on fibronectin expression, and finally by inhibition of ROS and PKC. The source of cellular ROS was further examined by inhibition of NADPH oxidase and mitochondrial metabolism.

Results. D-glucose increased dichlorofluorescein (DCF)-sensitive cellular ROS in HPMC in a dose-dependent manner. L-glucose did not induce ROS generation and cytochalasin B completely blocked high glucose-induced ROS generation, suggesting that glucose uptake, but not media hyperosmolality, is required in ROS generation in HPMC. PMA increased cellular ROS and fibronectin secretion. A single dose of H_2O_2 or H_2O_2 continuously generated by glucose oxidase fibronectin expression. Antioxidants trolox and catalase inhibited high glucose- and PMA-induced fibronectin mRNA and protein expression. Inhibition of PKC inhibited high glucose- and H_2O_2-induced fibronectin secretion. NADPH oxidase inhibitors (diphenyleneiodinium and apocynin) and an inhibitor of mitochondrial electron transport chain subunit I (rotenone) all effectively inhibited high glucose-induced cellular ROS generation and fibronectin secretion.

Conclusion. The present data demonstrate that high glucose increases cellular ROS in HPMC through activation of PKC, NADPH oxidase, and mitochondrial metabolism and that ROS, thus generated, up-regulate fibronectin expression by HPMC. ROS are not only downstream but also upstream signaling molecules to PKC and provide signal amplification in high glucose-induced fibronectin expression by HPMC. The present data imply that cellular ROS may be potential therapeutic targets in progressive accumulation of extracellular matrix in the peritoneal tissue of long-term peritoneal dialysis patients using high glucose-containing peritoneal dialysis solutions.

Key words: peritoneal dialysis, peritoneal fibrosis, oxidative stress, protein kinase C, NADPH oxidase, mitochondrial metabolism.

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Long-term peritoneal dialysis is associated with progressive increase in the thickness of peritoneal membrane, predominantly in the submesothelial compact collageneous zone [1]. The mechanisms involved in this structural change remain unclear but prolonged exposure of the membrane to high concentration of glucose may be one of them. High glucose up-regulates the expression of fibronectin [2–4], transforming growth factor-β1 (TGF-β1) [2, 3, 5, 6], and basic fibroblast growth factor (bFGF) [7] in human peritoneal mesothelial cells (HPMC). Peritoneal dialysis solutions containing high concentration of glucose and glucose degradation products stimulate procollagen III N-terminal peptide and TGF-β1 secretion by HPMC [8].

We [2] previously demonstrated that high glucose-induced up-regulation of fibronectin expression in HPMC is mediated by activation of protein kinase C (PKC) and this was confirmed by a recent study by Chan et al [3]. In glomerular mesangial cells, PKC mediates high glucose-induced fibronectin secretion [9], and stimulates collagen α1 (IV) transcriptional activity [10]. PKC is known to generate reactive oxygen species (ROS) [11, 12] and PKC-dependent activation of the reduced form of

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was recently suggested to be an important mechanism for increased oxidative stress in diabetes [13]. On the other hand, high glucose-induced PKC activation in glomerular mesangial [14, 15] and aortic endothelial cells [16] and in diabetic kidney [17] is mediated by ROS. ROS activate signal transduction cascade and transcription factors leading to up-regulation of genes and proteins involved in renal fibrosis in diabetes [18–20]. We, therefore, hypothesized that high glucose-induced PKC activation in HPMC may stimulate cellular ROS and ROS thus generated may in turn activate PKC and provide signal amplification in high glucose-induced fibronectin expression in HPMC.

In this regard, patients undergoing peritoneal dialysis are under increased oxidative stress possibly through increased ROS generation and suppressed antioxidative defense mechanisms [21, 22]. However, the role of ROS in peritoneal fibrosis and the molecular mechanism for high glucose-induced ROS generation in HPMC remain unclear. NADPH oxidase is known to be the most important source of receptor-mediated ROS generation in nonphagocytic cells [23, 24] and high glucose-induced ROS generation in HPMC may be through activation of NADPH oxidase. That ROS may also be generated in HPMC through mitochondrial metabolism is suggested by a recent study [25], which demonstrated mitochondrial DNA damage induced by glucose-containing peritoneal dialysis solutions.

In this study, we tested our hypothesis that ROS play a role in high glucose-induced fibronectin expression in HPMC. We visualized and quantified dichlorofluorescein (DCF)-sensitive ROS in HPMC stimulated with high glucose and phorbol 12-myristate 13-acetate (PMA), examined the effect of high glucose, PMA, and hydrogen peroxide (H_2O_2) on HPMC fibronectin secretion, and the effect of PKC inhibition and antioxidants on fibronectin secretion by HPMC. We also examined the effects of inhibition of NADPH oxidase and mitochondrial electron transport chain subunit to elucidate the mechanisms involved in high glucose-induced ROS generation in HPMC.

METHODS

All chemicals and tissue culture plates, unless otherwise stated, were obtained from Sigma Chemical Company (St. Louis, MO, USA) and Nalge Nunc International (Naperville, IL, USA), respectively.

HPMC culture

HPMC were isolated, cultured, and characterized according to the previously described method [2]. Human omentum was obtained from the consenting patients undergoing abdominal surgery or cesarean section. All experiments were performed using cells in the second or third passage.

Cells (2×10^5 cells/well) were cultured in 6-well culture plate, unless otherwise stated. For visualization of intracellular ROS, cells were cultured on cover glass coated with poly-L-lysine. Near-confluent HPMC were incubated with serum-free media for 24 hours to arrest and synchronize the cell growth. After this time period, the media were changed to fresh serum-free medium M199 containing different concentrations of glucose, 100 μmol/L H_2O_2, or 80 nmol/L PMA. In our preliminary experiments, H_2O_2 at 100 μmol/L induced cellular ROS to a similar extent by 100 mmol/L D-glucose. H_2O_2 was not cytotoxic to HPMC at 100 μmol/L, while H_2O_2 at 500 μmol/L was. In order to achieve a steady and continuous supply of H_2O_2 in the media, 10 mU/mL glucose oxidase were added to fresh serum-free media supplemented with 5.6 mmol/L glucose. This concentration of glucose oxidase continuously produces H_2O_2 for up to 48 hours without significant effect on lactate dehydrogenase (LDH) release.

To demonstrate that glucose-induced ROS generation in HPMC was specific for D-glucose uptake and metabolism, cells were pretreated with 0.2 μmol/L cytochalasin B (ICI Research Laboratories, Alderly Park, Cheshire, UK), a noncompetitive inhibitor of glucose transporter [26], or treated with L-glucose as an osmotic control. Cytochalasin B was cytotoxic in high dose as estimated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. However, absorbance at 0.2 μmol/L, used in this study, was 0.312 and was not significantly different from control (0.356) and 0.02 μmol/L (0.345).

To determine the importance of ROS on fibronectin mRNA and protein expression in HPMC, 500 μmol/L trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Aldrich Chemical Company, Milwaukee, WI, USA), a water soluble vitamin E, or 500 U/mL catalase was added 1 hour before the addition of high glucose, PMA, H_2O_2, or glucose oxidase. The dosages of antioxidants used in this study are the dosages that were found effective in inhibiting high glucose-induced generation of ROS. To investigate the interaction between PKC and ROS in high glucose-induced fibronectin expression, HPMC were stimulated with PMA in the presence and absence of antioxidants or with H_2O_2 in the presence and absence of PKC inhibition. One hundred nanomol/L diphenyleneiodinium chloride (DPI) and 100 μmol/L apocynin were used to evaluate the role of NADPH oxidase and 5 μmol/L rotenone to study the role of mitochondrial metabolism in high glucose-induced ROS generation and fibronectin secretion. The dosages of DPI, apocynin, and rotenone used in this study were the dosages that did not affect LDH release.

After incubation, intracellular ROS was visualized as described below. For immunoblot analysis or enzyme-linked immunosorbent assay (ELISA) for fibronectin, the media were collected and centrifuged to remove cell debris before used for assay. After removing the media, cells were lysed after washing with phosphate-buffered saline (PBS) and the concentration of cellular protein was measured using Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA, USA). Incubation of cells in control as well as high glucose in serum-free condition for up to 96 hours had no significant effect on cell viability as determined by LDH release.

Assay of cellular ROS

Cellular ROS production was measured by confocal microscopy according to the previously described method [27]. Briefly, confluent cells at 1 hour after stimulation with high glucose or 15 minutes after PMA were washed with Dulbecco's PBS and incubated in the dark for 15 minutes in Krebs-Ringer solution containing 5 μmol/L 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA) (Molecular Probes, Inc., Eugene, OR, USA). Culture dishes were transferred to a Leica DM IRB/E inverted microscope, equipped with a ×20 Fluotar objective and Leica TCS NT confocal attachment (Wetzlar, Germany), and the ROS generation was detected as a result of the oxidation of DCF (excitation, 488 nm; emission, 515 to 540 nm). The effect of DCF photo-oxidation was minimized by collecting the fluorescent image with a single rapid scan (line average, 4; total scan time, 5.2 seconds) and identical parameters, such as contrast and brightness, for all samples. The cells were then imaged by differential interface contrast microscopy. DCF-sensitive cellular ROS were also quantified by fluorescence-activated cell scan (FACS) (Becton Dickinson, Mountain View, CA, USA) after detaching HPMC with trypsin [28].

Real-time reverse transcription-polymerase chain reaction (RT-PCR) of fibronectin mRNA expression

Expression of fibronectin mRNA was assessed by real-time PCR using the SYBR Green System (iCycler Real-Time PCR Detection System; Bio-Rad Laboratories, Inc.) according to the previously described method [29–31] with some modification. Our previous Northern blot analysis demonstrated that high glucose or PMA significantly up-regulates fibronectin mRNA expression at 24 hours [2]. Total RNA from HPMC was extracted using Trizol reagent (Life Technologies BRL, Rockville, MD, USA) and converted into cDNA with Superscript First Strand Synthesis system for RT-PCR (Life Technologies BRL) according to the manufacturer's manual. All cDNA reactions were performed in a total volume of 20 μL. Two microliters of each sample was used to measure mRNA levels of fibronectin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The forward primer for fibronectin was 5′-CGAGAGTAAACCTGAAGCTG-3′ and the reverse primer for fibronectin was 5′-CC TTGTGTCCTGATCGTTTGC-3′. The forward primer for GAPDH was 5′-CCTGCACACCAACTGCTTA GC-3′ and the reverse primer for GAPDH was 5′-CCAGTGAGCTTCCCGTTCAGC-3′. The amplification was performed with the following time course: 94°C, 10 minutes and 40 cycles of 94°C, 1 minute; 58°C, 1 minute; and 72°C, 1 minute. Detection of the fluorescent products was carried out at the end of the 72°C extension period. Each sample was tested in triplicate. To confirm amplification specificity the PCR products were subjected to a melting curve analysis and subsequent agarose gel electrophoresis [29, 31]. Results were expressed as relative changes to control.

Immunoblot analysis of fibronectin protein

Immunoblot analysis was performed to determine the production and secretion of fibronectin into media at 48 hours after various experimental conditions. We previously demonstrated that high glucose or PMA significantly up-regulates fibronectin secretion at 48 hours [2]. Aliquots of conditioned media were mixed with sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol and heated at 95°C for 15 minutes. Samples were applied to 5% polyacrylamide gel and electrophoresed. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane using a transblot chamber with Tris buffer. Equal amount of samples were verified by ponceau S staining. Western blots were incubated with peroxidase-conjugated rabbit antihuman fibronectin (Dako A/S, Glostrup, Denmark) for 2 hours at room temperature, washed with Tris-buffered saline–Tween-20 for 1 hour. After washing, the membranes were incubated with enhanced chemiluminescence (ECL) detection reagents (Amersham Life Science, Little Chalfont, UK). Positive immunoreactive bands were quantified densitometrically and compared to controls.

ELISA

In some experiments, fibronectin secreted into the media was also quantitated by ELISA to confirm the results obtained from Western blot analysis. Aliquots of conditioned media were analyzed by a commercial competitive inhibition ELISA for fibronectin (Chemicon International, Temecula, CA, USA) according to the manufacturer's description. The assay is sensitive to 10 to 20 ng fibronectin/mL.

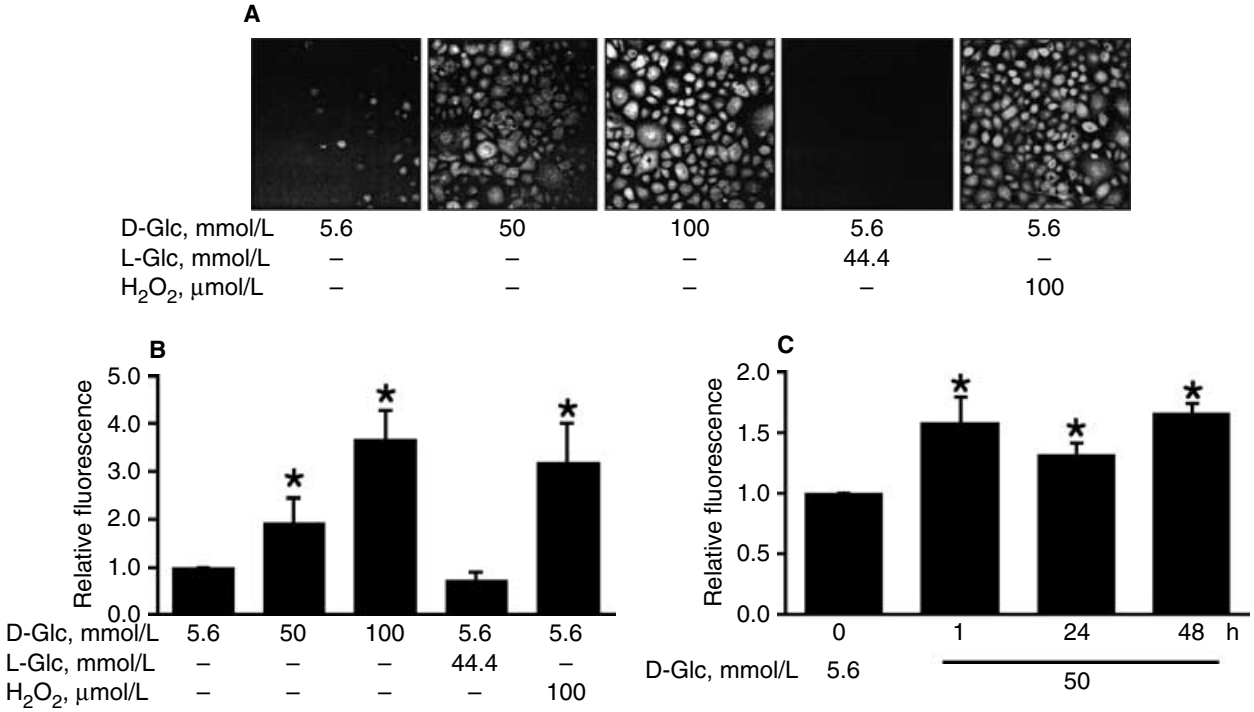

Fig. 1. Effects of D-glucose and exogenous H_2O_2 on cellular reactive oxygen species (ROS) in human peritoneal mesothelial cells (HPMC). After incubation of quiescent HPMC with 5.6 mmol/L, 50 mmol/L, and 100 mmol/L D-glucose (D-Glc) or 44.4 mmol/L L-glucose (L-Glc) + 5.6 mmol/L D-glucose for 1 hour or with 100 μmol/L H_2O_2 for 15 minutes, dichlorofluorescein (DCF)-sensitive cellular ROS were measured by confocal microscopy (*A* and *B*) as described in the text. Cellular ROS in HPMC cultured under 50 mmol/L D-glucose for 1, 24, and 48 hours were quantified by fluorescence-activated cell scan (FACS) (*C*). (A) Representative picture of DCF-sensitive cellular ROS by a confocal microscopy. (B) Fluorescent intensity relative to that of 5.6 mmol/L D-glucose (control). Values are expressed as mean ± SE of four to six experiments. $^*P < 0.05$ compared to control.

Analysis of data

All results are expressed as mean ± standard error (SE) with *N* as the number of experiments using cells from different donors. The mean values obtained from each group were compared by analysis of variance (ANOVA) with subsequent Fisher least significant difference method. In view of the small number of experiments, the Kruskal-Wallis test and the Mann-Whitney *U* test were also used. A P value < 0.05 was used as the criterion for a statistically significant difference.

RESULTS

High glucose increases cellular ROS in HPMC

Fifty millimolar D-glucose induced DCF-sensitive cellular ROS as early as 15 minutes and the increase was significant at 1 hour (Fig. 1) and remained significantly elevated at 48 hours (Fig. 1C) as compared with control. We chose to determine cellular ROS at 1 hour after glucose treatment in subsequent experiments. As summarized in Figure 1A and B, glucose induced cellular ROS in a dose-dependent manner. Fifty and 100 mmol/L D-glucose increased cellular ROS 1.9- and 3.7-fold, respectively, that of 5.6 mmol/L glucose as measured by confocal microscopy. Since glucose at high concentrations can induce cell dysfunction by hyperosmolality [6, 32], we used 50 mmol/L, but not 100 mmol/L glucose as the high glucose. Unlike D-glucose, addition of 44.4 mmol/L L-glucose into M199 containing 5.6 mmol/L D-glucose did not induce cellular ROS in HPMC. H_2O_2 100 μmol/L added to media resulted in cellular ROS to a similar extent by 100 mmol/L D-glucose. Cytochalasin B at 0.2 μmol/L completely blocked high glucose-induced cellular ROS (Fig. 2A).

Effects of antioxidants on high glucose-induced ROS in HPMC

Trolox at 500 μmol/L abolished high glucose-induced ROS generation in HPMC (Fig. 2B). High glucose-induced ROS generation was also effectively blocked by 500 U/mL, but not by 50 U/mL, of catalase, an enzyme that converts H_2O_2 into water and oxygen (Fig. 2B).

Effects of high glucose and H_2O_2 on fibronectin expression by HPMC

As we previously demonstrated [2], high glucose significantly increased fibronectin mRNA expression in HPMC at 24 hours (Fig. 3). Both trolox and catalase, at doses that inhibited intracellular ROS, effectively

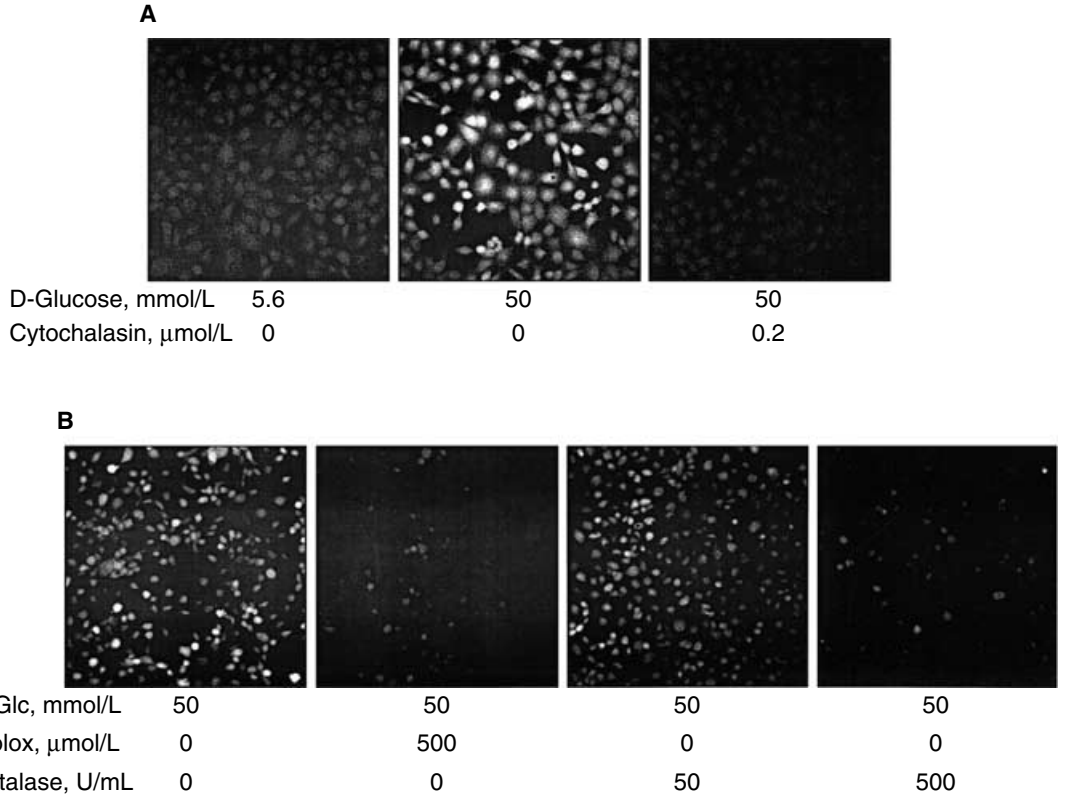

Fig. 2. Effects of cytochalasin B (*A*) and antioxidants (*B*) on high glucose-induced cellular reactive oxygen species (ROS) in human peritoneal mesothelial cells (HPMC). Quiescent HPMC were incubated with 50 mmol/L D-glucose in the presence or absence of 0.2 μmol/L cytochalasin B (A) or 500 μmol/L trolox and 50 and 500 U/mL catalase (B) for 1 hour and dichlorofluorescein (DCF)-sensitive cellular ROS were measured as described in the text. Each is a representative picture of four independent experiments.

inhibited high glucose-induced, but not basal, fibronectin mRNA expression.

High glucose-induced fibronectin mRNA expression was translated into up-regulation of protein (Fig. 4A). High glucose significantly increased fibronectin secretion by HPMC at 48 hours and this was effectively blocked by trolox. Figure 4B shows that single dose of H_2O_2 also significantly increased fibronectin secretion at 48 hours and this was effectively inhibited by trolox and catalase. Figure 4C demonstrates that continuous generation of H_2O_2 by 10 mU/mL glucose oxidase in the presence of 5.6 mmol/L glucose increased fibronectin secretion by HPMC comparable to the level induced by high glucose and significantly higher compared to control glucose. Antioxidants trolox and catalase effectively inhibited high glucose- and glucose oxidase-induced fibronectin secretion. Fibronectin secretion by control glucose was not inhibited by either trolox or catalase.

Interaction between PKC and ROS in high glucose-induced fibronectin protein secretion by HPMC

As shown in Figure 5A, PMA at 80 nmol/L increased intracellular ROS by twofold in HPMC at 15 minutes. Figure 5B shows that PMA also significantly increased fibronectin secretion at 48 hours as we previously demonstrated [2] and this was effectively inhibited by trolox and catalase.

Figure 6 demonstrates that both calphostin C, a PKC inhibitor, and PKC depletion by preincubating cells with PMA for 24 hours inhibited not only PMA-induced fibronectin secretion but also high glucose- and H_2O_2-induced, but not basal, fibronectin secretion.

Role of NADPH oxidase and mitochondrial metabolism in high glucose-induced cellular ROS generation and fibronectin protein secretion by HPMC

Figure 7A demonstrates that inhibitors of NADPH oxidase DPI and apocynin and an inhibitor of mitochondrial electron transport complex I rotenone inhibited high glucose-induced, but not basal, fibronectin secretion. DPI, apocynin, and rotenone all effectively inhibited high glucose-induced ROS generation in HPMC (Fig. 7B).

Figure 8 is a schematic representation of the mechanisms involved in high glucose-induced cellular ROS generation and the interaction between PKC and ROS in high glucose-induced fibronectin expression by HPMC.

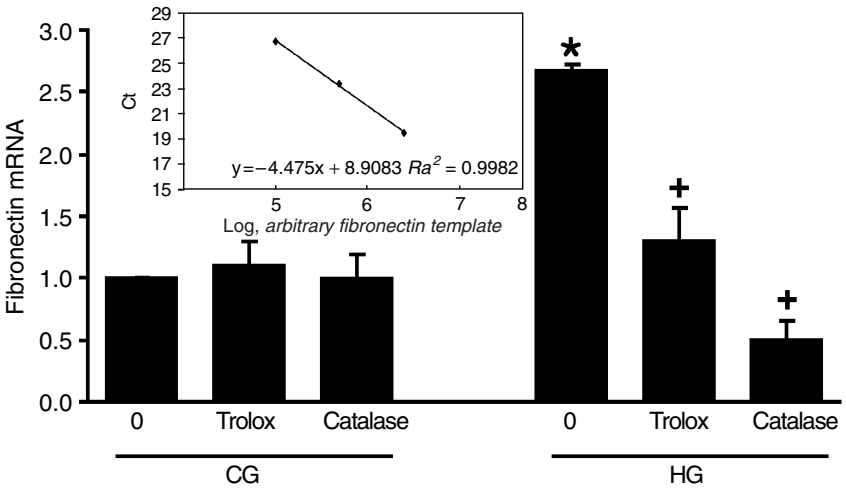

Fig. 3. Effects of antioxidants on high glucose-induced fibronectin mRNA expression in human peritoneal mesothelial cells (HPMC). After incubation of quiescent HPMC with 5.6 mmol/L (CG) or 50 mmol/L D-glucose (HG) in the presence or absence of trolox or catalase for 24 hours, total RNA was isolated and real-time polymerase chain reaction (PCR) was performed as described in the text. Inset graph demonstrates linear relationships between cycle threshold (Ct) and serial 1:10 dilutions of cDNA in sample. Data are expressed as mean ± SE fold increase that of control from four experiments. $^{*}P < 0.05$ compared to control; $^{+}P < 0.05$ compared to HG without antioxidant. CG, control glucose; HG, high glucose.

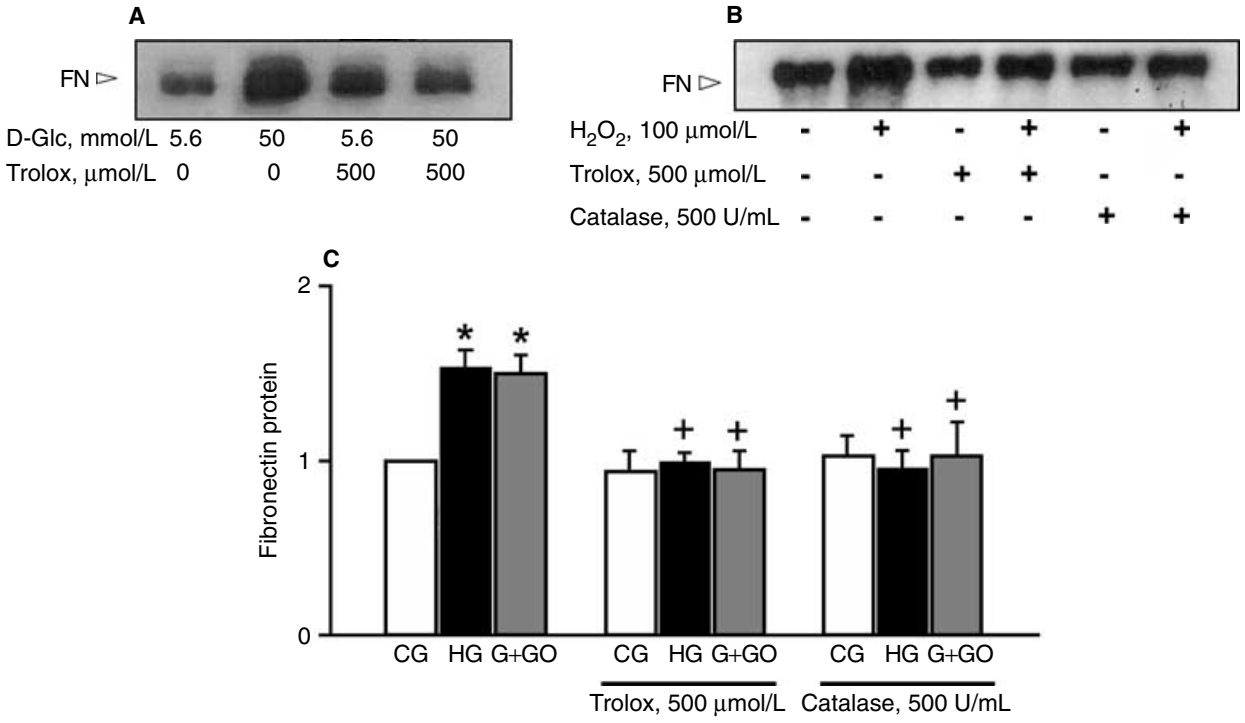

Fig. 4. Effects of antioxidants on high glucose- (*A* and *C*) and H_2O_2-induced (*B* and C) fibronectin protein secretion by human peritoneal mesothelial cells (HPMC). After incubation of quiescent HPMC with 5.6 mmol/L (CG) or 50 mmol/L D-glucose (HG), 100 μmol/L H_2O_2, or 10 mU/mL glucose oxidase (GO) + 5.6 mmol/L glucose in the presence or absence of trolox or catalase for 48 hours, aliquots of conditioned media were electrophoresed under reducing condition for Western blots (A and B) or enzyme-linked immunosorbent assay (ELISA) were performed (C) as described in the text. (A) Representative Western blot analysis from five experiments demonstrating the effects of trolox on HG-induced fibronectin (FN) secretion by HPMC. (B) Representative Western blot analysis from three experiments showing the effects of trolox and catalase on H_2O_2-induced FN secretion by HPMC. (C) H_2O_2 was continuously generated from glucose oxidase and glucose (G). FN was measured by ELISA. Data are expressed as mean ± SE fold increase that of control from four to seven experiments. FN produced by HPMC under serum-free CG media without antioxidants was 433 ± 140 μg/mg of cell protein. $^{*}P < 0.05$ compared to control; $^{+}P < 0.05$ compared to HG and G + GO without antioxidants.

DISCUSSION

In this study, we demonstrated that high glucose and PKC significantly increase DCF-sensitive cellular ROS, that H_2O_2 as well as PKC up-regulates fibronectin expression, that antioxidants inhibit high glucose- and PKC-induced fibronectin expression, and that inhibition of PKC suppresses high glucose- and H_2O_2-induced fibronectin expression in HPMC. Finally, we demonstrated that inhibition of NADPH oxidase and mitochondrial metabolism inhibited high glucose-induced ROS generation and fibronectin expression in HPMC.

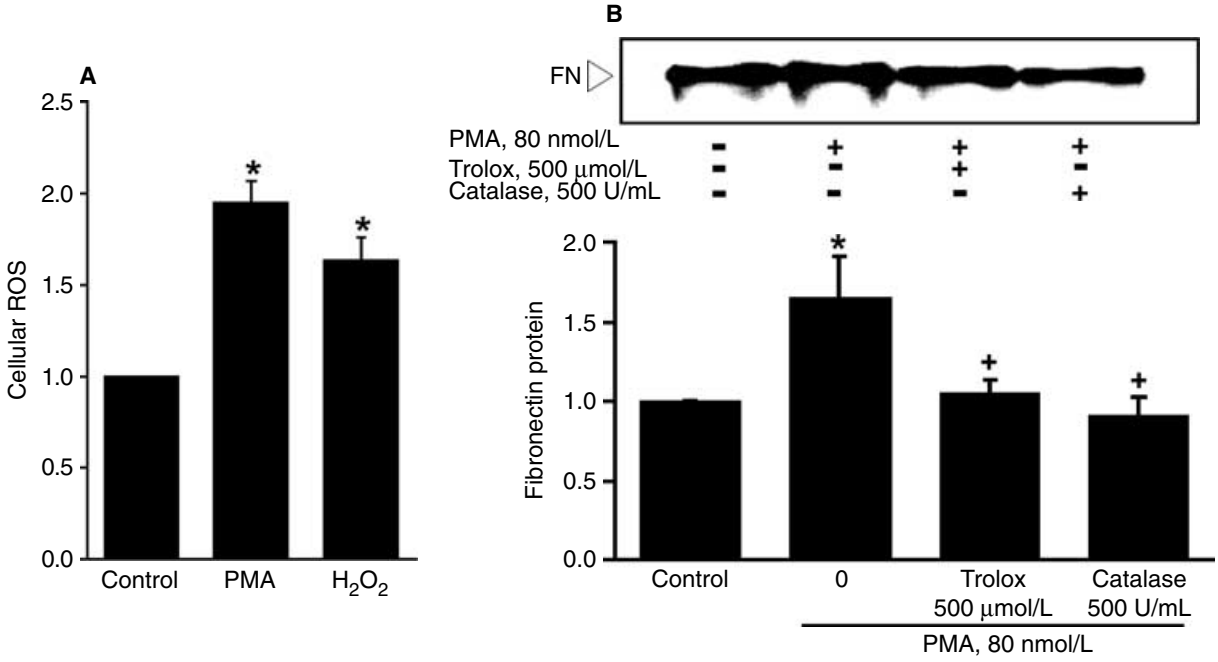

Fig. 5. Effects of phorbol 12-myristate 13-acetate (PMA) on cellular reactive oxygen species (ROS) generation (*A*) and effects of antioxidants on PMA-induced fibronectin secretion (*B*) by human peritoneal mesothelial cells (HPMC). (A) After incubation of quiescent HPMC with 80 nmol/L PMA or 100 μmol/L H_2O_2 for 15 minutes, cellular ROS was measured by fluorescence-activated cell scan (FACS). (B) After incubation of quiescent HPMC with 80 nmol/L PMA for 48 hours in the presence or absence of trolox or catalase, aliquots of conditioned media were electrophoresed under reducing condition and Western blots were performed for fibronectin (FN) as described in the text. Data are expressed as mean ± SE fold increase that of control from five experiments. $^*P < 0.05$ compared to control: $^+P < 0.05$ compared to PMA without antioxidants.

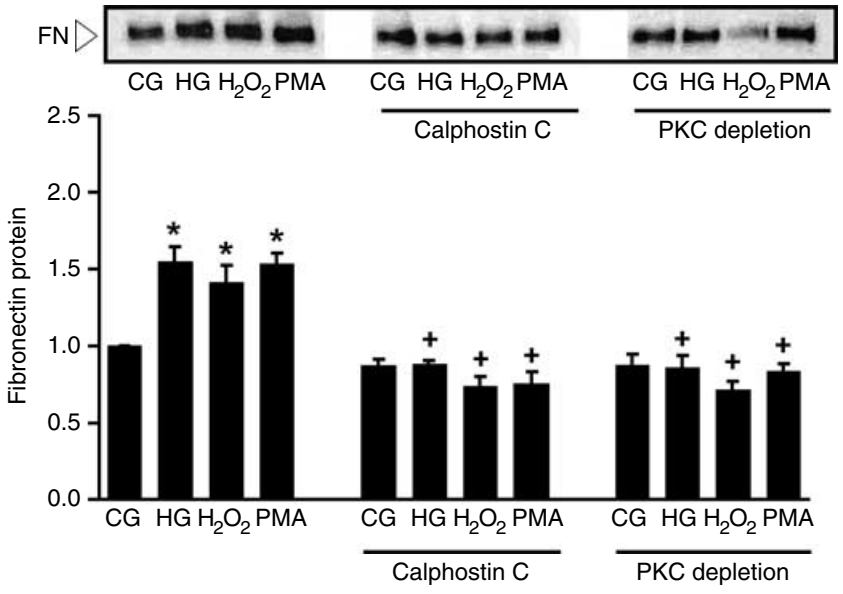

Fig. 6. Effects of protein kinase C (PKC) inhibition on phorbol 12-myristate 13 acetate (PMA)-, high glucose-, and H_2O_2-induced fibronectin secretion by human peritoneal mesothelial cells (HPMC). After incubation of quiescent HPMC with 5.6 mmol/L (CG), 50 mmol/L D-glucose (HG), 100 μmol/L H_2O_2, and 80 nmol/L PMA in the presence and absence of PKC inhibition for 48 hours, aliquots of conditioned media were electrophoresed under reducing condition and Western blots were performed for fibronectin (FN) as described in the text. Data are expressed as mean ± SE fold increase that of control from five experiments. $^*P < 0.05$ compared to CG; $^+P < 0.05$ compared to HG, H_2O_2, and PMA without PKC inhibition.

We [2] and others [3] have previously demonstrated that PKC mediates high glucose-induced fibronectin secretion by HPMC and this was reproduced in the present study. Antioxidants effectively inhibited PMA-induced fibronectin secretion by HPMC, suggesting that ROS are downstream-signaling molecules to PKC. On the other hand, inhibition of PKC suppressed H_2O_2-induced fibronectin secretion by HPMC, suggesting that PKC mediates ROS-induced fibronectin secretion. Indeed, ROS has been suggested as an upstream regulator of PKC under high glucose, since H_2O_2-induced fibronectin expression by rat glomerular mesangial cells is effectively inhibited by PKC inhibition or depletion [18] and blockade of mitochondrial superoxide overproduction inhibit PKC activation in vascular endothelial cells cultured under high glucose [16]. These observations suggest that

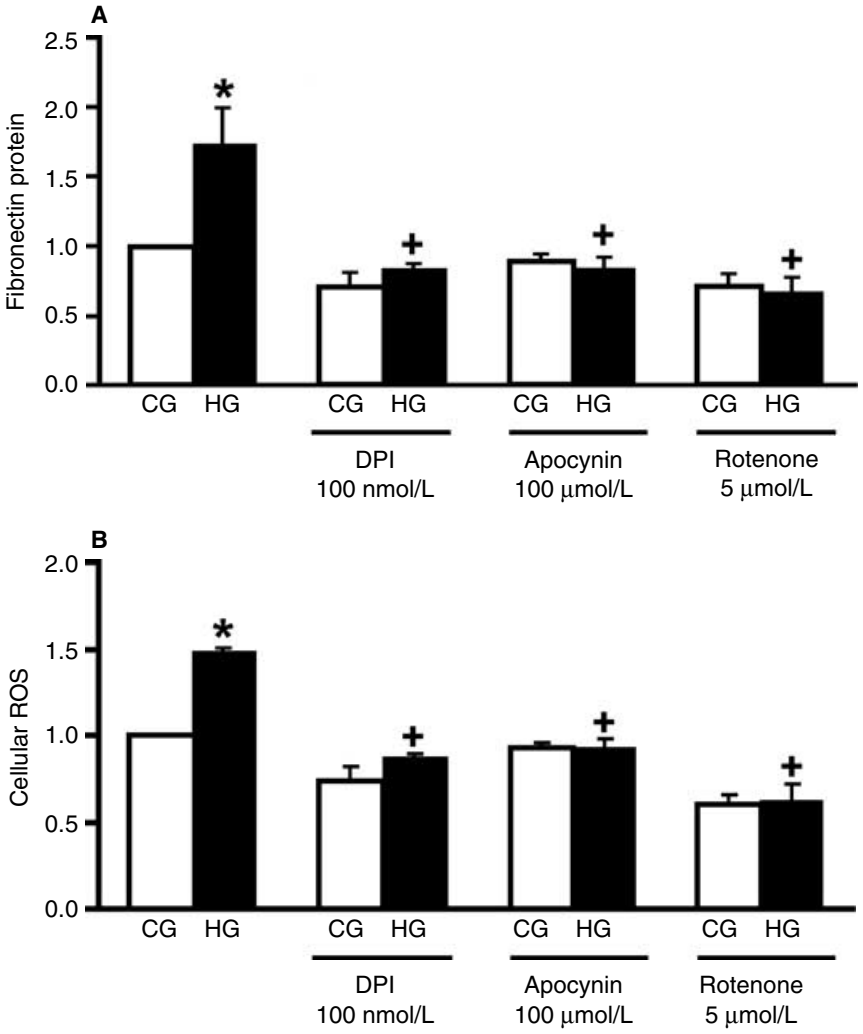

Fig. 7. Effects of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitors or an inhibitor of mitochondrial electron transport chain subunit on high glucose-induced fibronectin secretion (*A*) and cellular reactive oxygen species (ROS) generation (*B*) in human peritoneal mesothelial cells (HPMC). After incubation of quiescent HPMC with 5.6 mmol/L (CG) and 50 mmol/L D-glucose (HG) in the presence and absence of NADPH oxidase inhibitors diphenyleneiodinium chloride (DPI) 100 nmol/L or apocynin 100 μmol/L, or an inhibitor of mitochondrial electron transport chain subunit I rotenone 5 μmol/L for 48 hours (A) or for 1 hour (B), emzyme-linked immunosorbent assay (ELISA) for fibronectin was performed using aliquots of conditioned media (A) or dichlorofluorescein (DCF)-sensitive cellular ROS were measured by fluorescence-activated cell scan (FACS) (B). Data are expressed as mean ± SE fold increase that of control from five experiments. $^{*}P < 0.05$ compared to CG; $^{+}P < 0.05$ compare to HG without inhibition of NADPH oxidase or mitochondrial electron transport.

ROS can amplify PKC-induced signaling in high glucose-induced fibronectin expression by HPMC.

Unlike D-glucose, L-glucose did not induce cellular ROS in HPMC. A glucose transporter inhibitor cytochalasin B effectively inhibited high glucose-induced cellular ROS. Cultured primary HPMC constitutively express glucose transporters 1 and 3 and express sodium-dependent glucose transporter when differentiated [33]. These observations suggest that glucose uptake and metabolism, but not high osmolality, are required for high glucose-induced ROS generation in HPMC.

Based on the observations that DCF diacetate enters into cells and makes non-fluorescent 2, 7-dichcorofluoroscein (DCFH), which fluoresces when it reacts with hydroperoxides and/or H_2O_2 [34, 35], and that catalase effectively inhibited high glucose-induced ROS generation in the present study, H_2O_2 appears to be the main ROS generated by high glucose in HPMC. High glucose-induced H_2O_2 generation by rat peritoneal mesothelial cells have been demonstrated [36].

Although molecular mechanisms involved in high glucose-induced ROS generation are not clear, metabolism subsequent to glucose uptake, glucose autooxidation, and advanced glycation end products (AGEs) formation have been proposed as possible pathways. AGEs are produced by a process involving nonenzymatic modification of proteins by physiologic sugars and their reactive dicarbonyls such as glyoxal, 3-deoxyglucosone, and methylglyoxal. AGEs are independent risk factors for diabetic complications [37, 38] and peritoneal permeability changes in continuous ambulatory peritoneal dialysis [39, 40]. ROS are generated in the process of AGE formation and through AGE-AGE receptor binding and induce a wide range of cellular responses, including up-regulation of cytokines and hormones [41, 42]. It is unlikely that increase in cellular ROS early (1 hour) after high glucose is AGE-related, although it is possible that late (48 hours) increase may be related to AGE.

Glucose auto-oxidation is involved in high glucose-induced ROS generation in human endothelial cells [43], but not in rat and mouse mesangial cells [27]. In our preliminary study, 3-O-methyl-D-glucose at 50 mmol/L increased intracellular ROS 1.7-fold that of control

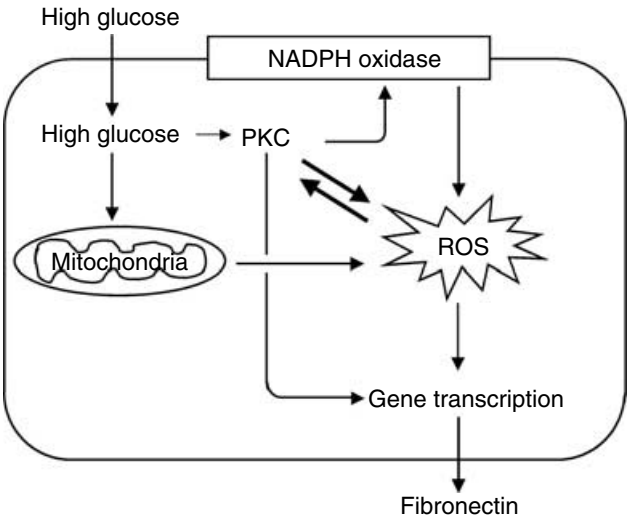

Fig. 8. Mechanism for high glucose-induced cellular reactive oxygen species (ROS) in human peritoneal mesothelial cells (HPMC). High glucose generates cellular ROS in HPMC through activation of protein kinase C (PKC), the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and mitochondrial metabolism. ROS are not only downstream but also upstream signaling molecules to PKC and provide signal amplification in high glucose-induced fibronectin secretion by HPMC.

glucose in HPMC (data not shown), suggesting possible involvement of glucose autooxidation in high glucose-induced ROS generation in HPMC.

PKC is activated as a result of glucose metabolism and mediates high glucose-induced fibronectin expression by HPMC [2]. PKC is well known to generate ROS [11, 12] and this was confirmed in our present study. In addition, antioxidants effectively inhibited PMA-induced fibronectin secretion, suggesting that PKC activation may play a role in high glucose-induced ROS generation and fibronectin up-regulation by HPMC. PKC-dependent activation of NADPH oxidase was recently suggested to be an important mechanism responsible for increased oxidative stress in diabetes [13]. In recent years, vascular NADPH oxidase has been intensively studied as a key mechanism for ROS generation [23, 24]. Present observation that both DPI and apocynin inhibited high glucose-induced fibronectin secretion as well as cellular ROS generation suggests that NADPH oxidase may also play a role in high glucose-induced ROS generation in HPMC as in vascular smooth muscle and endothelial cells [13]. Since DPI and apocynin are nonspecific inhibitors of NADPH oxidase, future studies utilizing specific inhibition of each subunit of NADPH oxidase are required.

Our observation that rotenone, an inhibitor of mitochondrial electron transport chain subunit I, inhibited high glucose-induced ROS generation and fibronectin secretion suggests that mitochondrial metabolism may be involved in high glucose-induced ROS generation in HPMC as in vascular endothelial cells [16]. A recent study by Ishibashi et al [25] demonstrated increased mitochondrial DNA damage in peritoneal mesothelial cells cultured under high glucose and glucose containing dialysate.

CONCLUSION

The present data provide evidence that high glucose-induced ROS generation plays an important role in fibronectin synthesis by HPMC. The present study also demonstrates that high glucose generates cellular ROS in HPMC through activation of PKC, NADPH oxidase, and mitochondrial metabolism. ROS are not only downstream but also upstream signaling molecules to PKC and provide signal amplification in high glucose-induced fibronectin expression by HPMC. These data imply that cellular ROS may be potential therapeutic targets in progressive accumulation of extracellular matrix in the peritoneal tissue of long-term peritoneal dialysis patients using high glucose-containing conventional peritoneal dialysis solutions.

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